

# In vivo targeted gene delivery to peripheral neurons mediated by neurotropic poly(ethylene imine)-based nanoparticles

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**Abstract:** A major challenge in neuronal gene therapy is to achieve safe, efficient, and minimally invasive transgene delivery to neurons. In this study, we report the use of a nonviral neurotropic poly(ethylene imine)-based nanoparticle that is capable of mediating neuron-specific transfection upon a subcutaneous injection. Nanoparticles were targeted to peripheral neurons by using the nontoxic carboxylic fragment of tetanus toxin (HC), which, besides being neurotropic, is capable of being retrogradely transported from neuron terminals to the cell bodies. Nontargeted particles and naked plasmid DNA were used as control. Five days after treatment by subcutaneous injection in the footpad of Wistar rats, it was observed that 56% and 64% of L4 and L5 dorsal root ganglia neurons, respectively, were expressing the reporter protein. The delivery mediated by HC-functionalized nanoparticles spatially limited the transgene expression, in comparison with the controls. Histological examination revealed no significant adverse effects in the use of the proposed delivery system. These findings demonstrate the feasibility and safety of the developed neurotropic nanoparticles for the minimally invasive delivery of genes to the peripheral nervous system, opening new avenues for the application of gene therapy strategies in the treatment of peripheral neuropathies.

**Keywords:** gene therapy, nonviral vector, neuron-targeted, peripheral neurons

## Introduction

Peripheral neuropathy is a clinical condition highly prevalent worldwide that still awaits efficient treatment options. Although peripheral neurons have intrinsic regenerative capacities, the provision of specific trophic factors to the injured neurons can result in a significant improvement in their survival and regeneration efficiency. Nonetheless, a reduced plasma half-life and adverse effects in nontargeted tissues as a consequence of systemic administration have limited their clinical application.<sup>1</sup> An alternative approach to achieve a controlled and targeted delivery of trophic factors in the nervous system is the use of gene-based therapies, in which a transgene encoding for a therapeutic protein is introduced into the target cells. However, the development of safe and efficient vectors for in vivo gene transfer remains a main challenge in order to attain the promise of gene therapy.

Gene transfer to the peripheral nervous system has been previously explored, mainly through the use of viral vectors, since some have natural neurotropism and are associated with high transduction efficiencies.<sup>2</sup> However, in spite of important advances toward the increased safety of viral vehicles, they still present risks regarding toxicity and immunogenicity.<sup>3</sup> In turn, nonviral gene delivery vectors have become attractive



alternatives to their viral counterparts, offering better safety profiles, although the majority continues to show low gene transfer efficiency.<sup>4</sup> Together with safety and efficiency issues, crucial requirements for in vivo gene therapy are the specific targeting of the desired cell population as a way to avoid off-target delivery and increase the transgene expression in the target tissue, assured by a minimally invasive administration route. Despite the wide number of nonviral gene delivery vectors proposed in the past, to the best of our knowledge, none has been developed to actively target peripheral neurons following a minimally invasive peripheral route of administration. Thus, such development is of significant interest not only for safety issues, but also to aid in the design of more clinically relevant therapeutic applications to treat neuropathic conditions. To this end, we have previously described the development of a nonviral gene delivery vector based on poly(ethylene imine) (PEI) targeted to peripheral neurons by its functionalization with a pegylated nontoxic carboxylic fragment of tetanus toxin (HC).<sup>5</sup> The HC fragment is neurotropic and can undergo active retrograde transport after peripheral administration.<sup>6,7</sup> Indeed, its potential as a therapeutic carrier has already been explored as a coupling agent to mediate the transport of proteins or viral vectors to neurons.<sup>8–10</sup> When functionalized with the pegylated HC fragment, the developed thiolated PEI-based nanoparticles (PEISH-HC) showed the ability to transfect primary cultures of dissociated dorsal root ganglia (DRG) neurons in a specific fashion.<sup>5</sup> Here, we aimed to evaluate the in vivo performance of these targeted nanoparticles, assessing their capacity to retrogradely access and transfect lumbar DRG neurons after a peripheral and minimally invasive administration in the animal's footpad. We hypothesize that after footpad injection, the nanoparticles contact the sensorial terminals, are internalized in a cell-specific fashion, are transported toward the DRGs that innervate the injection site, and lead to the local expression of the transgene.

## Materials and methods

### Materials

Branched PEI (25 kDa, Sigma-Aldrich Co., St Louis, MO, USA) was thiolated with 2-iminothiolane (Sigma-Aldrich Co.) and purified as previously described.<sup>5</sup> The thiolated PEI (PEISH) was dissolved at 1 mg/mL in a 5% (w/v) glucose solution (pH 7.4) and stored at  $-80^{\circ}\text{C}$  until further use. The plasmid DNA (pDNA) encoding for GFP and  $\beta$ -galactosidase (pVI-VO1-GFP-LacZ, 8.4 kb, Invivogen, San Diego, CA, USA) was used throughout the study. Plasmid was produced in a DH5 $\alpha$  competent *Escherichia coli* strain transformed with

the respective plasmid. Subsequently, DNA purification was performed using an endotoxin-free Maxiprep kit following the manufacturer's instructions (GenElute, Sigma-Aldrich Co.). The HC fragment was produced recombinantly using the BL21 *E. coli* strain. The plasmid encoding for the HC fragment was a kind offer from Prof Neil Fairweather (King's College, UK). The HC production in the BL21 *E. coli* strain and purification was performed as previously described.<sup>11</sup> The obtained HC fragment was additionally covalently linked to a bi-functional poly(ethylene glycol) (PEG) spacer. Briefly, a bi-functional 5 kDa PEG (JenKem Technology USA, Plano, TX, USA) bearing an N-hydroxysuccinimide and a maleimide end group was used as indicated by the manufacturer, at a 2.5 PEG/HC protein molar ratio.<sup>5</sup>

### Nanoparticles' preparation

Nanoparticles were prepared as previously described with an N/P molar ratio of 3 (N/P – moles of primary amine groups [N] of PEI to moles of DNA phosphate groups [P]) and a final concentration of 7.5  $\mu\text{g}$  pegylated HC per 2  $\mu\text{g}$  of pDNA.<sup>5</sup> Briefly, the nanoparticle core was formed by mixing, while vortexing, equal volumes of pDNA and PEISH solution in 5% (w/v) glucose in water (pH 7.4). Complexes were left to form for 15 minutes at room temperature. Subsequently, pegylated HC fragment (reactive to thiol moieties by a maleimide terminal group in the 5 kDa PEG) was added to the complex mixture at a final concentration of 7.5  $\mu\text{g}$  per 2  $\mu\text{g}$  of pDNA and ternary complexes were left to form for 24 hours at room temperature. Prior to use, the nanoparticle dispersion was concentrated to a final pDNA concentration of 500  $\mu\text{g}/\text{mL}$  in 5% (w/v) glucose aqueous solution (pH 7.4) using a 30 kDa cutoff filter (Amicon Ultra, EMD Millipore, Billerica, MA, USA).

### Nanoparticles' physicochemical characterization

PEISH-based nanoparticles with and without HC functionalization were characterized in terms of size, polydispersity index (Pdi), and zeta potential using a Zetasizer Nano Zs (Malvern Instruments, Malvern, UK). The Smoluchowski model was applied for zeta potential determination and cumulant analysis was used for mean particle size determination. Ten  $\mu\text{g}$  of pDNA was used to prepare the tested formulations. All measurements were performed in triplicate at  $25^{\circ}\text{C}$ . The morphology of the PEISH-based nanoparticles was evaluated by transmission electron microscopy (TEM). Briefly, 10  $\mu\text{L}$  of the nanoparticle suspension was placed on a grid, treated with sodium phosphotungstate, and observed in a TEM Zeiss 902 A. To assess the distribution of the

pegylated HC fragment on the surface of the nanoparticle core, a parallel experiment was performed in which HC fragment was coupled to Qdot® 800 ITK™ carboxyl quantum dots (Molecular Probes, Eugene, OR, USA) before its complexation with the thiolated nanoparticles. The resulting nanoparticles were adsorbed on a grid and imaged by TEM, without any further treatment.

## Animals and in vivo nanoparticles' administration

All animal experiments were carried out with the permission of the local animal ethics committee Instituto de Biologia Molecular e Celular (IBMC)-Instituto de Engenharia Biomédica (INEB), Associated Laboratory, Porto, Portugal) in accordance with the EU Directive (2010/63/EU) and Portuguese law (DL 113/2013). The experimental protocol was approved by the ethics committee of the Portuguese official authority on animal welfare and experimentation (Direção-Geral de Alimentação e Veterinária). A total of 34 male 4-month old Wistar rats, with an average weight of 350–400 g were used in this study. Animals were randomly divided into three groups as follows: A) pDNA group (subcutaneous injection of pDNA in 5% (w/v) glucose aqueous solution (pH 7.4), n=6); B) PEISH group (subcutaneous injection of PEISH nanoparticles in 5% (w/v) glucose aqueous solution (pH 7.4), n=14); and C) PEISH-HC group (subcutaneous injection of HC-functionalized PEISH nanoparticles in 5% (w/v) glucose aqueous solution (pH 7.4), n=14). For nanoparticles' administration, animals were anesthetized by the inhalation of isoflurane and the site of administration was disinfected with ethanol. Nanoparticle or pDNA dispersions were administered unilaterally in left footpad (150 µL of dispersion corresponding to 75 µg of pDNA) using a 25-gauge needle (Hamilton, Bonaduz, Switzerland). At 3 and 5 days post-injection, animals were sacrificed by a lethal intraperitoneal injection of 20% (v/v) sodium pentobarbital. In group A, and per time point, the tissue samples of two animals were processed for gene expression analysis by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and the tissue sample of one animal was processed for GFP expression detected by immunohistochemistry. In groups B and C, five and two animals were used, respectively. Tissues were collected and snap-frozen or fixed in 4% (v/v) buffered formalin for total RNA extraction or histological analysis, respectively. The following tissues were collected: posterior footpads, lymph nodes that drain the back limb region (ie, popliteal and inguinal), whole blood, sciatic nerves, L1–L4 ventral roots (VR), L4–L5 DRGs, heart, liver, spleen, kidneys, and lungs.

## RNA extraction and real-time RT-PCR

Total RNA was extracted from designated tissues using the PureLink RNA Mini kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Reverse transcription was obtained from 500 ng of total RNA employing the Superscript First-Strand Synthesis System (Thermo Fisher Scientific). For real-time quantification of *GFP* mRNA levels, the synthesized complementary DNAs were amplified in duplicate in an iCycler iQ5 (Bio-Rad Laboratories Inc., Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories Inc.). The following gene sequences were designed and synthesized: *GFP* sense primer: 5'-CTGTCCAACCTGGCAGACCATTACC-3', anti-sense primer: 5'-TTCCTGCTGCTGTCACAAACTCC-3'; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) sense primer: 5'-TTCACACCCATCACAAACAT-3', anti-sense primer: 5'-GCCATCAACGACCCCTTCAT-3'. At the end of the PCR cycling, correspondent melting curves were performed in order to ascertain the amplification of a single product and the absence of primer dimer formation. *GAPDH* gene was used as an internal control to normalize the variability in expression levels. The relative *GFP* mRNA expression levels in treatment groups were calculated relative to the control naked pDNA group using the 2- $\Delta\Delta C_T$  method described by Livak and Schmittgen, normalized with the average expression of *GAPDH*.<sup>12</sup>

## Histological analysis

Formalin-fixed tissues were processed and embedded in paraffin to prepare 4 µm paraffin sections. Dewaxed tissue sections were prepared for hematoxylin and eosin staining (ie, footpads) or for immunohistochemistry to detect the expression of GFP protein in the DRGs. The hematoxylin and eosin-stained footpad sections were used to qualitatively analyze the inflammatory response in the injection site as a result of the nanoparticle administration. The immunodetection of GFP protein in DRGs was used to calculate the in vivo transfection efficiency of DRGs mediated by PEI-based nanoparticles. Polyclonal rabbit anti-GFP (1:500, A11122, Thermo Fisher Scientific) and secondary antibody peroxidase polymer-labeled anti-rabbit (Dako Denmark A/S, Glostrup, Denmark) were used. The antibody reaction was revealed with diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) and tissue sections were counterstained with hematoxylin. To determine the transfection efficiency of PEISH-HC nanoparticles, the number of GFP positive L4 and L5 DRG neurons were counted in each 150 µm section for each sample. This corresponds to 5–6 sections and, on average, 1,000 neurons counted per DRG.

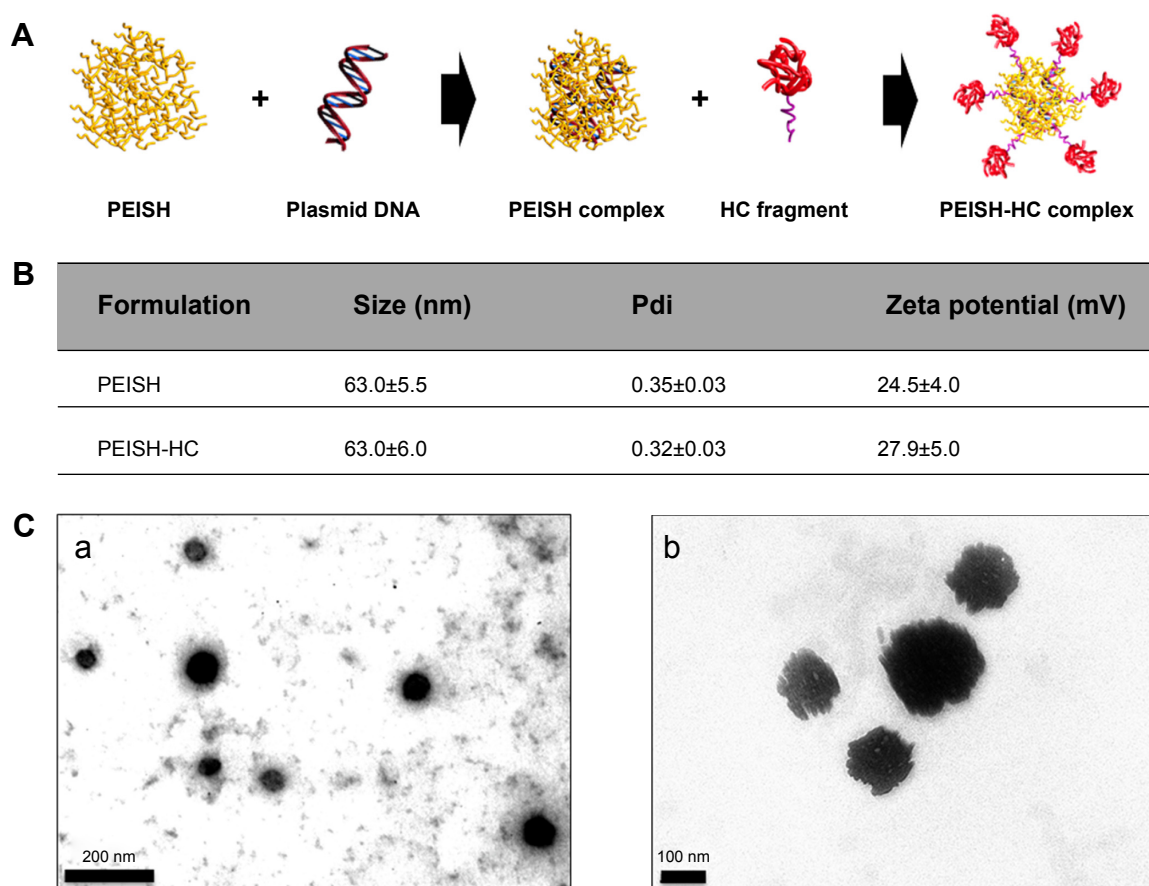
## Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as mean  $\pm$  standard deviation. D'Agostino and Pearson omnibus normality tests were used to test whether data obeyed a Gaussian distribution. The non-parametric Mann–Whitney *U*-test was performed to compare treatment groups. A result with  $P < 0.05$  was considered statistically significant.

## Results and discussion

The strategy followed for the development of neurotropic PEISH-based nanoparticles has been previously described by us.<sup>5</sup> As a first step, the PEISH is complexed with a pDNA suspension resulting in a naked complex. In the second step, the thiol-decorated complex is functionalized with the HC fragment via a 5 kDa bi-functional PEG linker, which is

thiol-reactive, resulting in the formation of a targeted complex (Figure 1A). Here, we explored the in vivo performance of the proposed PEISH neurotropic nanoparticles, prepared at an N/P ratio of 3. This formulation was chosen as it has been previously shown, based on molecular recognition force spectroscopy studies,<sup>13</sup> to possess an optimal targeting profile toward neuronal cells in vitro, as well as mediating targeted sensory neuron cell transfection in dissociated DRG primary cultures.<sup>5</sup> The tested nanoparticle formulations – the neurotropic nanoparticles based on PEISH-HC and the nontargeted PEISH based nanoparticles – were fully characterized in terms of size, Pdi, zeta potential, and morphology. In Figure 1B the presented table summarizes the physicochemical characterization of both formulations. The mean size diameter values obtained were  $63.0 \pm 5.5$  nm for naked nanoparticles and  $63.0 \pm 6.0$  nm for PEISH-HC nanoparticles, with the respective Pdi values  $0.35 \pm 0.03$  and  $0.32 \pm 0.03$ . The mean diameter of



**Figure 1** PEISH-based nanoparticle characterization.

**Notes:** (A) Scheme of nanoparticle synthesis. Modified from Oliveira H, Fernandez R, Pires LR, et al; Targeted gene delivery into peripheral sensorial neurons mediated by self-assembled vectors composed of poly(ethylene imine) and tetanus toxin fragment c; *J Control Release*; 143; 350–358; Elsevier; Copyright © 2010.<sup>5</sup> (B) Physicochemical characterization of PEISH-based nanoparticles at N/P ratio of 3 ( $n=3$ , mean  $\pm$  SD). (C) TEM photomicrographs of sodium phosphotungstate counterstained PEISH-HC nanoparticles (a) and HC-quantum dots functionalized nanoparticles (b).

**Abbreviations:** PEISH, thiolated poly(ethylene imine); Pdi, polydispersity index; N/P, moles of primary amine groups (N) of poly(ethylene imine) to moles of DNA phosphate groups (P); SD, standard deviation; TEM, transmission electron microscopy; HC, carboxylic fragment of tetanus toxin.



PEISH-HC nanoparticles is not significantly different than the naked nanoparticles showing that HC-functionalization does not influence the size of the nanoparticles. Zeta potential values obtained for naked and PEISH-HC nanoparticles were  $24.5 \pm 4.0$  and  $27.9 \pm 5.0$  mV respectively, indicating a cationic surface charge of nanoparticles with a good colloidal stability. The morphology of both nanoparticle formulations was assessed by TEM. In Figure 1C(a) a photomicrograph depicts PEISH-HC nanoparticles, being a representative image of the observed nanoparticle populations. These exhibited a spherical shape with no signs of aggregation. In Figure 1C(b), one can observe that the HC moieties grafted to the nanoparticles core are disposed at the surface of the nanoparticles, supporting the previously gathered evidences that the presence of the HC fragment at the nanoparticle surface confers the targeting features to these nanoparticles.

A single dose of naked pDNA, PEISH, or PEISH-HC nanoparticle dispersion in 5% (w/v) glucose aqueous solution (pH 7.4), carrying 75  $\mu$ g of pVIVO1-GFP-LacZ pDNA, was injected in the left footpad of adult Wistar rats. Previous studies regarding the peripheral administration of cationized gelatin-based nanoparticles reported transgene expression from 60 hours to 6 days post-posterior rat footpad injection.<sup>14</sup> In order to assess the ability of developed nanoparticles to efficiently access DRG neuron cell bodies after peripheral administration in the footpad, we evaluated the *GFP* transgene expression in the sciatic nerves, VR, and DRGs at 3 and 5 days post-administration.

In the case of animals treated with pDNA alone, the *GFP* expression was not detected in the sciatic nerve, VR, and DRG either at 3 or 5 days post-administration (data not shown).

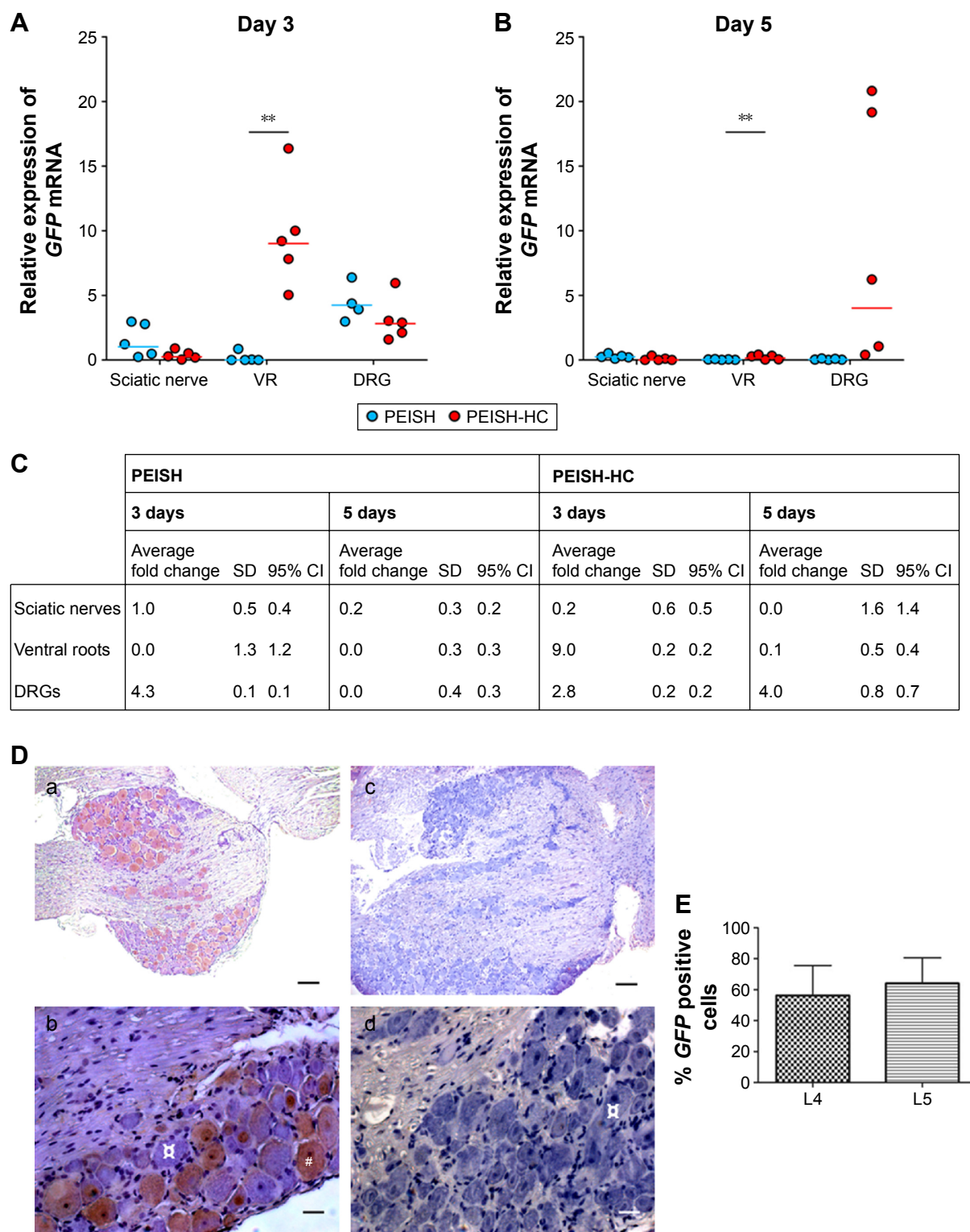
With regard to PEISH nanoparticles, at 3 days post-administration, a 1 and 4.3 average fold increase in *GFP* expression levels (relative to the expression levels in naked pDNA control group) could be detected in the sciatic nerves and DRGs, respectively (Figure 2A, C). Conversely, at day 5, no *GFP* expression was observed in the analyzed tissues (Figure 2B, C). Animals treated with the PEISH-HC nanoparticles showed a 9 and 2.8 average fold increase in *GFP* expression at the VR and DRGs at day 3, respectively (Figure 2A, C). Over the course of time, the *GFP* expression levels in DRGs further increased to four-fold (Figure 2B, C). These results proved that the transgene could be detected in DRGs as early as 3 days after footpad injection, and suggest that HC-grafted nanoparticles were retrogradely transported within the rate of fast active retrograde axonal transport (100–250 mm per day),<sup>15</sup> as passive diffusion could not account for the covered distance. Indeed, it has been shown that the nerve length from the toe to

the dorsal root entry was in the range of 17 cm in an adult rat,<sup>16</sup> what implies an active retrograde transport of the nanoparticles in order to reach the DRG in the observed period of time. At day 5, the transgene expression levels in DRG increased in relation to day 3 and, additionally, immunohistochemistry analysis at this time point showed that the transgene reporter protein could be detected (Figure 2D(a) and (b)).

Based on these results, we propose that the HC-functionalized nanoparticles were internalized at neuron terminals and followed retrograde fast axonal transport to the sensorial neuron cell bodies where they accumulated and led to transgene expression. Previous studies reported the ability of naked PEI-based nanoparticles to be retrogradely transported after subcutaneous administration in the footpad.<sup>14</sup> Our results are in accordance with these data, showing that naked PEISH-based nanoparticles were also able to deliver the *GFP* gene into DRGs, but they were not as efficient as their targeted counterparts and did not persist over time or resulted in reporter protein translation at detectable levels (Figure 2D(c) and (d)).<sup>14,17,18</sup>

One of the major limitations identified in nonviral gene delivery vectors is their inefficient transfection capacity. Here, we have shown that the developed neuron-targeted PEISH-HC nanoparticles were able to mediate the transfection and protein expression of 56% and 64% of L4 and L5 DRG neurons at 5 days post-administration (Figure 2E). The L4 and L5 DRG neurons are the ones which contribute more to the sciatic nerve that innervates the footpad.<sup>19</sup> A previous study regarding the subcutaneous footpad administration of recombinant adeno-associated serotype 6 viruses reported a transduction efficiency of L4 DRG neurons at less than 10%, at 3 weeks post-administration.<sup>20</sup> Also, the subcutaneous injection of cationized gelatin-based nanoparticles resulted in a transfection efficiency of approximately 10% of L4 and L5 DRG neurons.<sup>14</sup> These results highlight the improved transfection efficiency achieved in this work by means of active targeting toward peripheral neurons.

As important as the ability to reach the target tissues, is the capacity to reduce off-target distribution. In this sense we evaluated the transgene biodistribution profile, mediated by naked pDNA, PEISH, or PEISH-HC nanoparticles, upon subcutaneous administration, by assessing *GFP* mRNA expression in the following non-neuronal tissues: injected paws, left popliteal and inguinal lymph nodes (which drain the injected paws), whole blood, heart, liver, spleen, kidneys, and lungs. At 3 days post-injection, animals administered with pDNA alone revealed *GFP* gene expression solely in the injected paws (Figure 3A). However, at day 5, the transgene expression was detected in the majority of the tissues evaluated (Figure 3B). In PEISH-treated animals, the *GFP* transgene



**Figure 2** Reporter gene expression in neuronal tissues.

**Notes:** Relative GFP gene expression in neuronal tissues was evaluated at 3 (**A**) and 5 days (**B**) post-administration of PEI-based nanoparticles in the left footpad. (**C**) Statistical data characterization (n=5). (**D**) GFP protein expression in lumbar DRGs from animals treated with PEISH (c, d) or PEISH-HC nanoparticles (a, b) at 5 days post-administration. (**E**) Transfection efficiency of lumbar L4 and L5 DRG neurons mediated by PEISH-HC nanoparticles at day 5 post-administration. Mean  $\pm$  SD. (**A** and **B**) The GFP mRNA expression levels were normalized by GAPDH mRNA expression levels in each tissue sample and represented as relative to the GFP expression in naked pDNA control group. We considered the sciatic nerves, VR, and L4–L5 DRG as neuronal tissue. Each dot represents one animal. \*\*Denotes  $P < 0.01$ ; lines represent the geometric mean. (**D**) #Indicates a GFP positive cell; □ indicates a GFP negative cell. Scale bars = 100  $\mu$ m (a, c) or 25  $\mu$ m (b, d).

**Abbreviations:** PEI, poly(ethylene imine); PEISH, thiolated poly(ethylene imine); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; pDNA, plasmid DNA; VR, ventral roots; DRG, dorsal root ganglia; SD, standard deviation; CI, confidence interval; HC, carboxylic fragment of tetanus toxin.

could be detected in the injected paws, lymph nodes, heart, and liver at 3 and 5 days post-administration, and additionally in blood and spleen at 3 days post-administration (Figure 3). In contrast with these observations, animals treated with HC-functionalized PEISH nanoparticles revealed a restricted expression of *GFP* mRNA in non-neuronal tissues. As illustrated in Figure 3, the transgene expression was only detected in the injected paws and lymph nodes at both time points, and in the blood at 5 days post-administration. Moreover, the *GFP* gene expression levels in paws and lymph nodes were considerably lower in animals treated with PEISH-HC nanoparticles at both time points (Figure 3C, D). These results further support that HC-functionalized nanoparticles are able to spatially limit the transgene expression, in comparison with the nontargeted nanoparticles and naked pDNA, significantly reducing the possibility of unwanted off-target effects.

To further assess the impact of the local administration of the developed vectors, we evaluated the inflammatory response in the footpad at 3 and 5 days post-administration. In the first hours post-administration, independently of the tested formulation, animal footpads presented a moderate swelling indicative of a mild inflammatory reaction as a consequence of the injection, but 24 hours later the rat footpads regained their normal appearance and the animals showed normal use of the limb. Histologic analysis of the footpad at 3 and 5 days post-injection showed infiltration of inflammatory cells in the injection site (Figure 4). However, no relevant differences were observed between treatment groups, and the general behavior of animals under the tested conditions indicated that the developed systems do not induce a harmful effect, with neither footpad sensitization nor significant animal weight variation (data not shown) being observed, suggesting their safety.

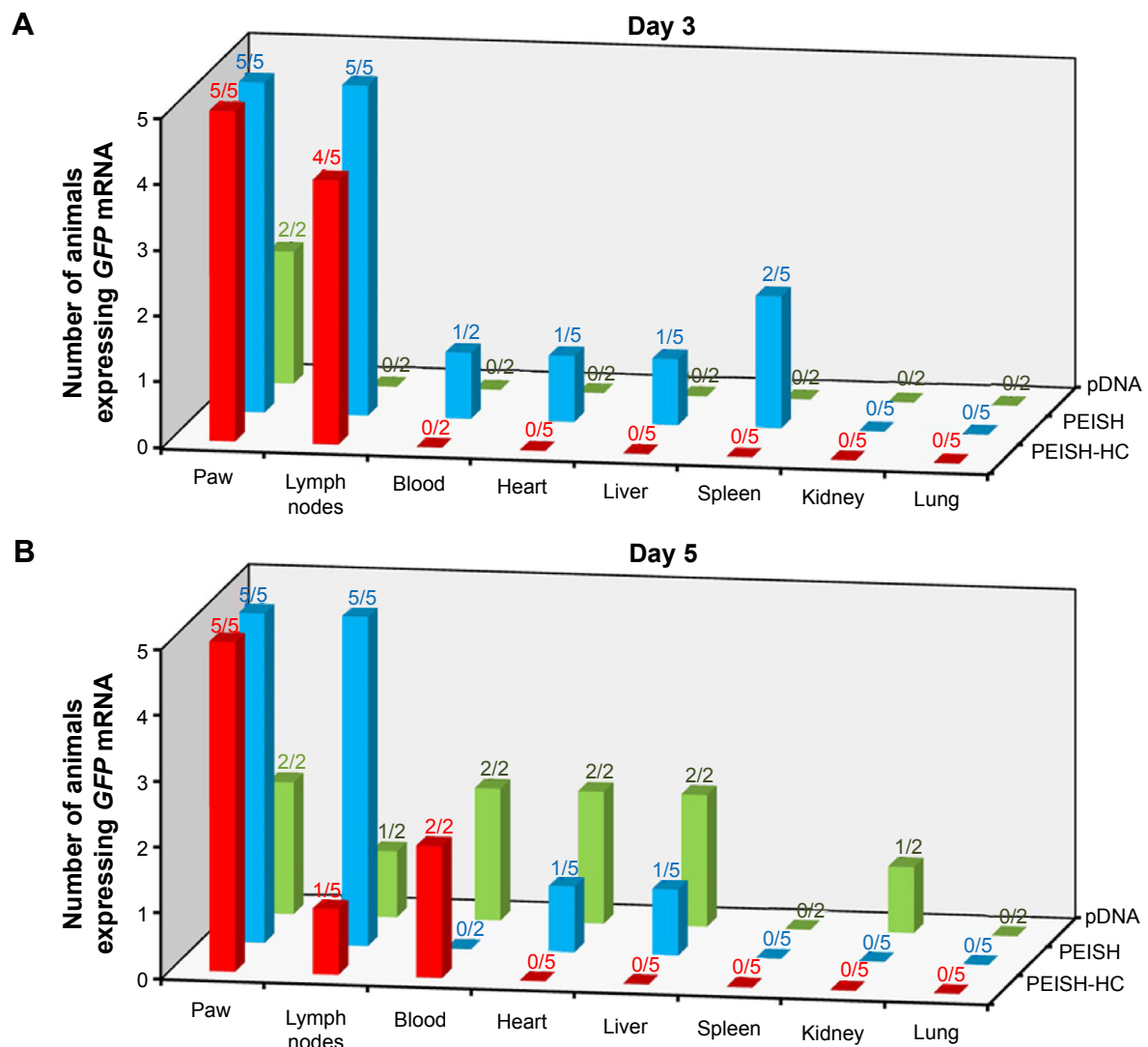
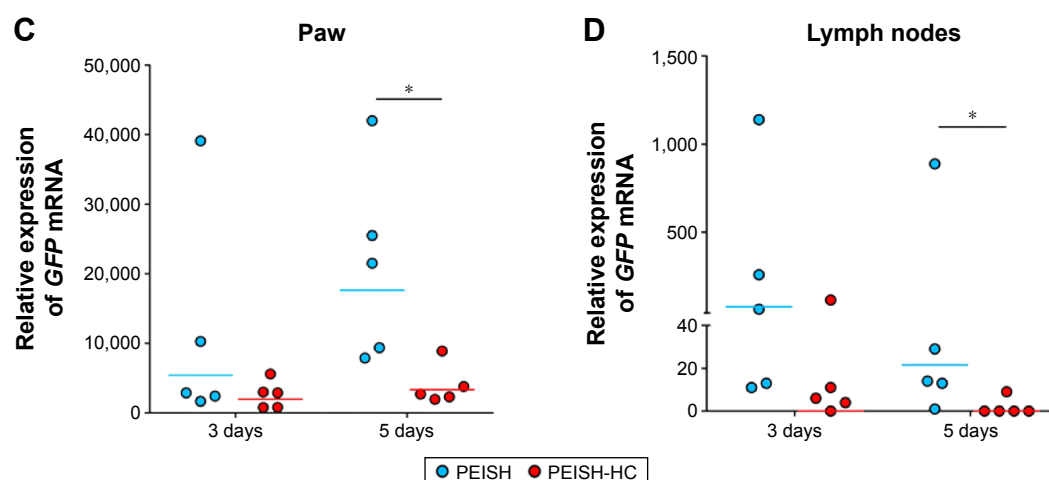


Figure 3 (Continued)





**Figure 3** Reporter gene expression in non-neuronal tissues.

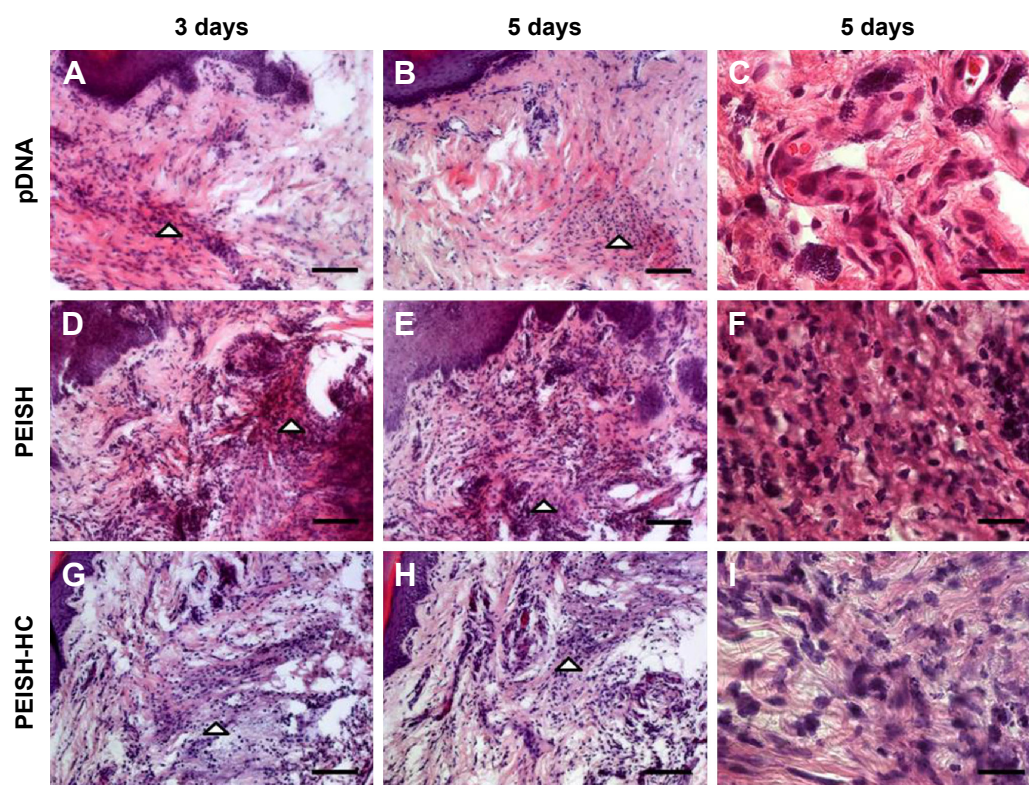
**Notes:** Firstly, GFP mRNA expression profile was qualitatively evaluated in non-neuronal tissues at 3 (A) and 5 (B) days post-administration. Relative GFP gene expression in paw (C) and lymph nodes (D) at 3 and 5 days post-administration of PEI-based nanoparticles in the left footpad. (A and B) For each tissue, over the columns, the number of animals expressing GFP mRNA in relation to the total number of animals evaluated is indicated (ie, 1/5 indicates one animal expressing GFP mRNA in a total of five animals analyzed at the same time point). (C and D) \*Denotes  $P < 0.05$ ; lines represent the geometric mean.

**Abbreviations:** pDNA, plasmid DNA; PEI, poly(ethylene imine); PEISH, thiolated poly(ethylene imine); HC, carboxylic fragment of tetanus toxin.

## Conclusion

This study demonstrates the efficient and targeted delivery of a reporter gene mediated by HC-modified PEISH-based nanoparticles to DRG sensorial neurons through a peripheral administration in the footpad. Likewise, these neurotropic

nanoparticles can be used to carry plasmids encoding for any relevant therapeutic gene in the nervous system, such as genes encoding for the neurotrophins NGF<sup>21</sup> and BDNF,<sup>5</sup> or pain-relief associated genes like *PENK*.<sup>22</sup> Such a targeted, versatile, and minimally invasive approach may prove to be



**Figure 4** Inflammatory response in the injection site.

**Notes:** Representative images of footpad subcutaneous tissue at 3 days (A, D, and G, scale bars = 100  $\mu$ m) and 5 days (B, E, and H, scale bars = 100  $\mu$ m; and C, F, and I, scale bars = 20  $\mu$ m) post-administration of naked pDNA (A–C), PEISH nanoparticles (D–F) or PEISH-HC nanoparticles (G–I). Arrowheads indicate the presence of infiltrating inflammatory cells.

**Abbreviations:** pDNA, plasmid DNA; PEISH, thiolated poly(ethylene imine); HC, carboxylic fragment of tetanus toxin.



useful for exploring new therapeutic strategies valuable for the treatment of sensory neuropathy, by stimulating sensory axon regeneration and/or preventing neuronal degeneration in the DRG, as well as for the relief of pain states resulting from various clinical conditions.

## Acknowledgments

We acknowledge Dr Teresa Summavielle (i3S – Instituto de Investigação e Inovação em Saúde/IBMC – Instituto de Biologia Molecular e Celular) and Dr Meriem Lamghari (i3S – Instituto de Investigação e Inovação em Saúde/INEB – Instituto de Engenharia Biomédica,) for their precious help regarding the in vivo study design and optimization; Dr Fani Neto, Dr António Avelino, and Dr Sara Adães (FMUP – Faculdade de Medicina da Universidade do Porto) for their help in tissue isolation; and Dr Rui Fernandes for the help with TEM analysis. This work was financed by FEDER – Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 – Operational Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through Fundação para a Ciência e a Tecnologia (FCT)/Ministério da Ciência, Tecnologia e Inovação in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274). Cátia DF Lopes acknowledges FCT for her PhD scholarship (SFRH/BD/77933/2011).

## Disclosure

The authors report no conflicts of interest in this work.

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